December 2007

Stable cell lines expressing high levels of the herpes simplex virus type 1 LAT are refractory to caspase 3 activation and DNA laddering following cold shock induced apoptosis

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Published in *Journal of General Virology* **88** (2007), 236-241; DOI 10.1099/vir.0.82066-0

Submitted March 24, 2006; accepted August 29, 2006.

Short Communication

OTK18, a zinc-finger protein, regulates human immunodeficiency virus type 1 long terminal repeat through two distinct regulatory regions

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Abstract : It has previously been shown by our laboratory that OTK18, a human immunodeficiency virus (HIV)-inducible zinc-finger protein, reduces progeny-virion production in infected human macrophages. OTK18 antiviral activity is mediated through suppression of Tat-induced HIV-1 long terminal repeat (LTR) promoter activity. Through the use of LTR-scanning mutant vectors, the specific regions responsible for OTK18-mediated LTR suppression have been defined. Two different LTR regions were identified as potential OTK18-binding sites by an enhanced DNA–transcription factor ELISA system; the negative-regulatory element (NRE) at –255/–238 and the Ets-binding site (EBS) at –150/–139 in the LTR. In addition, deletion of the EBS in the LTR blocked OTK18-mediated LTR suppression. These data indicate that OTK18 suppresses LTR activity through two distinct regulatory elements. Spontaneous mutations in these regions might enable HIV-1 to escape from OTK18 antiretroviral activity in human macrophages.

The control of human immunodeficiency virus (HIV) type 1 entry, reverse transcription, integration, expression and production is operated by distinct molecular mechanisms, which have been targeted by antiretroviral therapy. Among these mechanisms, nuclear factors, such as NF-κB and 45/39kD CCAAT/enhancer-binding protein-β (C/EBPβ) isoforms, are important in regulating HIV-1 expression (Griffin *et al.*, 1989; Henderson *et al.*, 1996). In addition, several transcription factors can repress virus transcription, often by binding to specific sequences contained in the virus long terminal repeat (LTR) (Pataca *et al.*, 1988; Subler *et al.*, 1994; Ray & Srinivas, 1997; Weiden *et al.*, 2000; Cicala *et al.*, 2002; Hayes *et al.*, 2002). Recently, we have shown that the transcriptional factor OTK18 is induced by and suppresses HIV-1 infection in mononuclear cells (Carlson *et al.*, 2004a). OTK18 is classified as a transcription factor as it contains 13 C_2H_2-type DNA-binding zinc-finger motifs (Saito *et al.*, 1996). C_2H_2 zinc-finger motifs are capable of binding to a wide range of DNA sequences, including the HIV-1 LTR (Wu *et al.*, 1995; Isalan *et al.*, 2001). We have demonstrated previously that one putative antiviral mechanism of OTK18 involves direct suppression of the LTR (Carlson *et al.*, 2004a). Mutational analyses revealed that the suppressive activity of OTK18 lies within aa 26–89. This region has homology to a family of zinc-finger proteins containing the Krüppel-associated box (KRAB) motif, a repression domain encoded by numerous transcription factors. Pengue *et al*. (1995) found that the KRAB domain fused to HIV-1 Tat was able to repress basal HIV-1 promoter activity in HeLa cells. This finding was supported further by the demonstration that a KRAB-containing tetracycline-binding protein can suppress HIV-1 replication through randomly integrated tetracycline-response elements within the HIV-1 genomic sequence (Herchenroder *et al.*, 1999). In addition, Reynolds *et al*. (2003) reported that a genetically engineered KRAB domain containing a C_2H_2-type zinc-finger protein could suppress Tat-mediated HIV-1 LTR activity, thereby making it an attractive candidate for antiretroviral therapy (Reynolds *et al.*, 2003). In this context, OTK18 is the first KRAB-containing C_2H_2 zinc-finger protein expressed endogenously in macrophages that has antiviral activity. Interestingly, OTK18 is expressed specifically in the cytosol of brain mononuclear phagocytes in severe HIV-1 encephalitis and may serve as a ‘surrogate’ marker for HIV-1-associated

dementia (Carlson *et al.*, 2004b). Elucidation of OTK18-binding sequences within the HIV-1 LTR is critical for the molecular characterization of OTK18 antiviral activity.

In this study, we employed all 27 LTR promoter linker-scanning mutants fused to the luciferase gene to screen for potential OTK18 response elements in the LTR (–453/+18) of the HXB2 clone (Zeichner *et al.*, 1991). The linker-scanning mutants consecutively replaced 18 bp of wild-type sequence with an *Nde*I–*Xho*I–*Sal*I(NXS) polylinker (CATATGCTCGAGGTCGAC) across the U3 and R regions. Human embryonic kidney 293 cells (10^5 cells per well on 24-well plates; Fisher Scientific) were co-transfected with the LTR-scanning mutant luciferase vectors (300 ng), Tat1–72 expression vector (pSV2Tat72, 50 ng) (Subramani *et al.*, 1981), a *Renilla* luciferase reference construct (pTK-RL, 50 ng) and the OTK18 expression vector (pcDNA-OTK18, 1 μg) by using GenePorter (Gene Therapy Systems) as described previously (Carlson *et al.*, 2004a). Forty-eight hours after transfection, cells were collected and luciferase activity was measured by using a luminometer (Berthold Systems Inc.) using a Dual-Luciferase kit (Promega). As shown in Table 1, we identified six regions (A3, A8, A10, A12, B1 and B6, corresponding to –417/–400, –327/–310, –291/–274, –255/–238, –237/–220 and –147/–130), which were resistant to the OTK18-mediated gene suppression in the presence of Tat. These codes correspond to the original codes by Zeichner *et al*. (1991).

The binding of OTK18 to each of these six regions was tested by a novel DNA–transcription factor ELISA, which is about tenfold more sensitive than conventional electromobility gel-shift analysis (EMSA). Development of such a technique was necessary, as binding of OTK18 to double-stranded oligonucleotides corresponding to the regions was too weak to be examined by conventional EMSA (data not shown). For that purpose, we generated a baculovirus expressing 6xHis-Express-tagged full-length OTK18 by in-frame insertion of the full-length OTK18 gene into the pBlueBacHis2A vector (Invitrogen) at the *Bam*HI site. The resultant vector (pBlueBacHis2A-OTK18) was inserted into Bac-N-Blue *Autographa californica* multiple nucleopolyhedrovirus DNA by homologous recombination according to the manufacturer's protocol. Infection of Sf9 insect cells with the optimized titre of OTK18 baculovirus resulted in expression of recombinant OTK18 at 84 h post-infection, detected as a 75 kDa protein (data not shown). OTK18 protein was collected specifically from the nuclear-extract fraction, which we used for subsequent experiments as control nuclear extract.

The DNA–transcription factor ELISA method was originally described by Reynolds *et al*. (2003) and is available commercially as the colorimetric ELISA TransAM kit (Active Motif). To enhance the sensitivity of the original protocol, we incorporated luminol-based conversion of hydrogen peroxidase activity to chemiluminescence by using SuperSignal ELISA Pico chemiluminescent substrate (Pierce) instead of conventional chromogenic development. As shown in Figure 1, the colorimetric ELISA shows a statistically significant difference between a non-specific oligonucleotide (control) and the HIV-1 LTR oligonucleotide A8 in the presence of OTK18-containing nuclear extract from 0.5 to 5 μg input (Figure 1a), but the signal-to-noise (S/N) ratio is 1.31–1.41 and Z′ factors are –5.64 to 0.045. In the case of our luminescence system, there is statistical significance between two groups from 0.5 to 5 μg input (Figure 1b), with an S/N ratio of 1.83–2.59 and Z′ factors of –0.88 to 0.85, which was calculated as described by Zhang *et al*. (1999). As a higher S/N ratio and Z′ factor indicate suitability of the assay system, we conclude that our luminescence system is superior to the colorimetric system, and we chose 2 μg nuclear extract for the following experiment.

The OTK18-binding activity to each element and its sensitivity to cold probe ranging from 1x to 100x was tested (Figure 1c). The oligonucleotide pairs used for double-stranded DNA probes are A3 [biotin-(Nx100)-CCTTGATCTGTGGATCTA and TAGATCCACAGATCAAGG], A8 [biotin-(Nx100)-TGGATGGTGCTACAAGCT and AGCTTGTAGCACCATCCA], A10 [biotin-(Nx100)-GAAGTTAGAAGAAGCCAA and TTGGCTTCTTCTAACTTC], A12 [biotin-(Nx100)-CTTGTTACACCCTGTGAG and CTCACAGGGTGTAACAAG], B1 [biotin-(Nx100)-CCTGCATGGAATGGATGA and TCATCCATTCCATGCAGG], B6 [biotin-(Nx100)-TCCGGAGTACTTCAAGAA and TTCTTGAAGTACTCCGG] and EBS [biotin-(Nx100)-CATCCGGAG and CTCCGGATG]. OTK18 binding was specific, as significant binding occurred only in the presence of OTK18-infected cell lysate and not in the presence of lysis buffer alone or uninfected cell lysate (Figure 1d). Whilst regions A3, A8 and B1 demonstrated significant binding to OTK18, none of them was outcompeted significantly by cold probes in a dose-dependent manner (Figure 1d). Only the A12 and EBS elements showed significant binding to OTK18, which was outcompeted by cold probes. The B1 element showed OTK18 binding, but was not outcompeted by cold probe, and B6, which contains partial EBS sequence, had no binding activity. In addition, none of the binding was outcompeted by up to 100-fold excess of single-stranded oligonucleotides corresponding to the binding sequence, suggesting its specificity to double-stranded DNA (data not shown). These data indicate that the A12 and EBS elements are potential specific OTK18-binding sites on the HIV-1 LTR.

The EBS is known to be an important response element for the cooperative interaction of Ets-1 with the upstream stimulatory factor (USF)-1 in HIV-1 enhancer activity (Sieweke *et al.*, 1998). As the B6 region does not cover

Table 1. Suppression of LTR linker-scanning mutants by OTK18

Region	Location	OTK18 suppression*	Transcriptional element
A1	−453/−436	++	
A2	−435/−418	++	
A3	−417/−400	−	
A4	−399/−382	++	
A5	−381/−364	+	Site A
A6	−363/−346	+	AP-1
A7	−345/−328	++	AP-1, site B, NRE
A8	−327/−310	−	NRE
A9	−309/−292	+	NRE
A10	−291/−274	−	NRE, NFAT-1
A11	−273/−256	++	NRE, NFAT-1, IL-2
A12	−255/−238	−	NRE, IL-2
B1	−237/−220	−	NRE, IL-2
B2	−219/−202	++	NRE
B3	−201/−184	++	NRE
B4	−183/−166	++	C/EBP II
B5	−165/−148	++	USF-1
B6	−147/−130	−	Ets, LEF-1
B7	−129/−112	++	LEF-1, C/EBP I
B8	−111/−94	++	NF-κB, HIVEN86A, EBP-1
B9	−93/−76	NA	NF-κB, HIVEN86A, EBP-1
B10	−75/−58	NA	Sp1
B11	−57/−40	NA	Sp1
B12	−39/−22	NA	TATA
C1	−21/−4	NA	LBP-1, UBP-1
C2	−3/+15	+	LBP-1, UBP-1
C4	−105/−81	+	NF-κB

*Suppression of pSVTat72-mediated LTR-luciferase expression by OTK18; −, + and ++ denote no suppression, 50–79 % suppression and 80–99 % suppression, respectively. NA, No detectable LTR activation by Tat and no suppression by OTK18.

EBS completely, we created an HXB2-derived LTR luciferase vector lacking EBS (–150/–139) (pLTRΔEBS-Luc) with a modified QuikChange II site-directed mutagenesis kit (Stratagene) using oligonucleotide pairs and tested its promoter activity in the presence/absence of HIV-1 Tat and OTK18 in 293 cells (Figure 2). Unexpectedly, pLTRΔEBS-Luc showed enhanced luciferase activity in the presence of Tat and OTK18 (second column) compared with Tat alone (first column). Tat-activated luciferase activity of the original LTR-Luc, on the other hand, was suppressed significantly by OTK18 (Figure 2b). This suggested that EBS is a critical element for the OTK18-mediated LTR suppression and that OTK18 has a dual regulatory function in the HIV-1 LTR, dependent on specific binding regions (see below). As the proximal promoter region between nucleosomes nuc0 and nuc1, where EBS is located, has been established as a critical regulatory region, EBS is an important site for the suppressive effect of OTK18. All of the above data indicate that EBS (–150/–139) is the primary OTK18 response element on the HIV-1 LTR.

We have shown previously that the transcriptional factor OTK18 suppresses both HIV-1 Tat-mediated LTR activation *in vitro* and HIV-1 replication in human monocyte-derived macrophages (MDMs) (Carlson *et al.*, 2004a). However, the response element for OTK18 suppression has not been determined. We have shown that OTK18 interacts with two distinct regions on the HIV-1 LTR, the NRE and EBS regions, by using LTR-scanning mutants and EMSA and through more defined subcloning/deletional analyses. Multiple regions of the NRE have been reported to downregulate HIV transcription (Garcia *et al.*, 1987). Specifically, NRE (–182/–153) contains a binding site for the nuclear factors USF (Sawadogo *et al.*, 1988), NFIL-6 (Tesmer *et al.*, 1993) and human GATA-3 (Yang & Engel, 1993; Galio *et al.*, 1999). Furthermore, NRE (–220/–160) binds to nuclear matrix proteins and inhibits NF-κB activity (Hoover *et al.*, 1996). However, no proteins have been identified that bind to NRE (–255/–238). Thus, OTK18, to the best of our knowledge, is the first cellular protein to be shown to interact with this specific region of the NRE and suppress the HIV-1 LTR.

A number of mutations were reported at this region in LTRs derived from human genomic DNA of HIV-1-infected patients. Estable *et al*. (1996) reported LTR-proximal sequences from 42 HIV-1-infected cases ranging from stage I to IV patients (World Health Organization

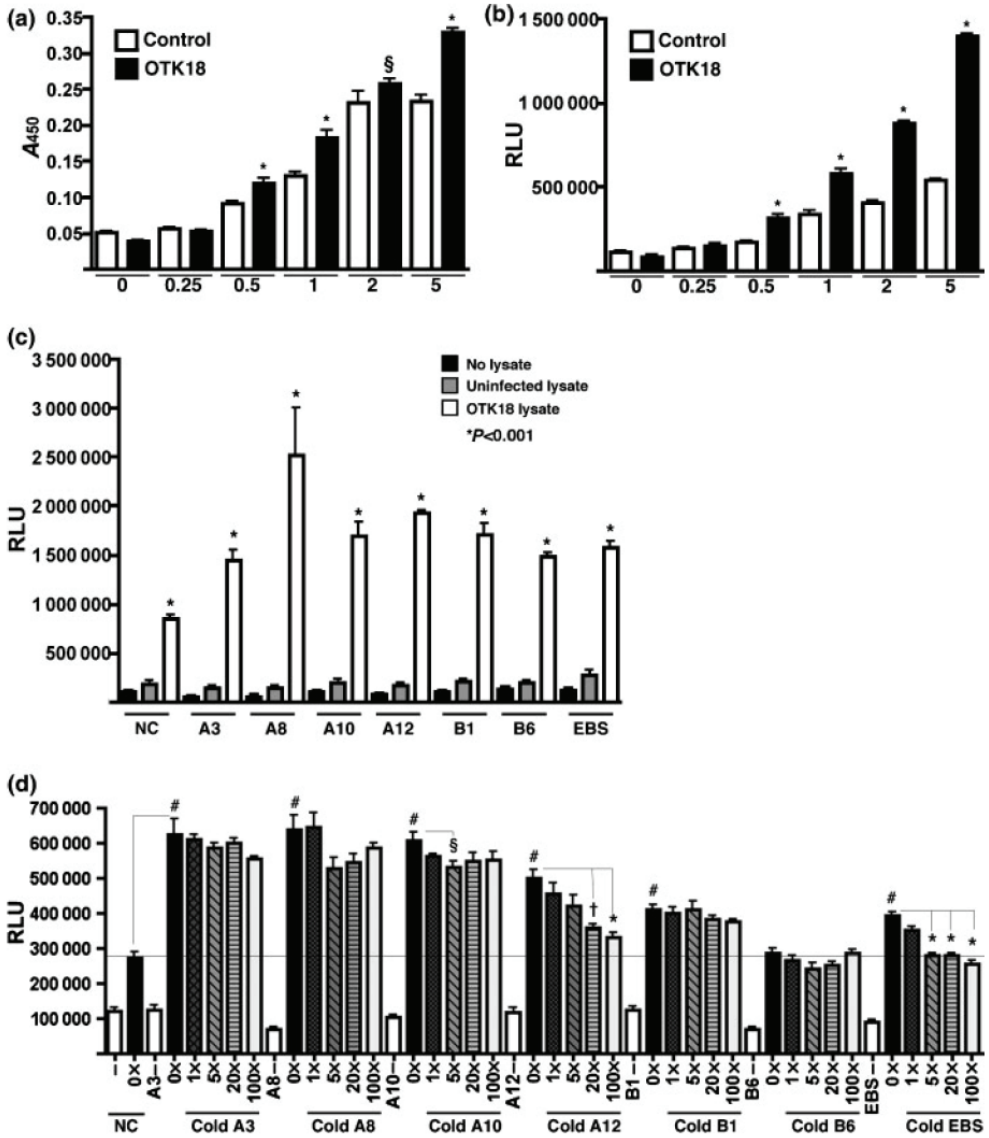

Figure 1. DNA ELISA screening for binding of OTK18 to HIV-1 LTR regions. (a, b) Improved chemiluminescent method for DNA–transcription factor ELISA. Sensitivity of two methods, conventional colorimetry (a) and chemiluminescence (b), was compared by using varying amounts of OTK18 protein extracts bound to a negative-control oligonucleotide (empty bars) or a double-stranded oligonucleotide corresponding to the A8 region of the HIV-1 LTR region (filled bars). Statistical analysis was performed by using one-way ANOVA to compare binding of each amount of extract with the binding of the negative-control oligonucleotide for the corresponding amount of extract (**P*<0.001; §*P*<0.05). (c) Six regions of the HIV-1 LTR (A3–B6 and EBS) were screened for specific binding of the OTK18 protein. Binding was compared in the presence of lysis buffer alone (no lysate, filled bars), uninfected Sf9 cell lysate (uninfected lysate, shaded bars) or OTK18 baculovirus-infected lysate (OTK18 lysate, empty bars). Only the OTK18 lysate showed significant binding (**P*<0.001 compared with no lysate or uninfected lysate). (d) Binding was tested in the presence of no extract (–, empty bars), extract alone with no competing oligonucleotide (0x, filled bars), 1x excess of the corresponding LTR oligonucleotide (checked bars), 5x excess oligonucleotide (diagonally hatched bars), 20x excess oligonucleotide (horizontally hatched bars) or 100x oligonucleotide (shaded bars). Statistical analysis was performed by using one-way ANOVA to compare binding of each oligonucleotide with no competitor present to the corresponding no-extract condition (#*P*<0.001). Statistical analysis was also performed by using one-way ANOVA to compare binding of the competition conditions with the no-competitor condition for the same oligonucleotide (†*P*<0.01; #*P*<0.001). NC, Negative control; RLU, relative luciferase units.

staging I–IV). Although they concluded that the Ets core sequence (ATCCG) was highly conserved, 25 of a total of 60 LTR sequences from the 42 cases were mutated in the Ets-1 element. The Ets core sequence was highly conserved in non-B subtypes (De Arellano *et al.*, 2005). We have also examined the available LTR depository at the Los Alamos HIV sequence database for LTRs of A, B, C and D subtypes. The conservation of ETS core sequence (ATCCG) was 19/19 (100 %, A), 45/48 (94 %, B), 73/74 (97 %, C) and (90 %, D).

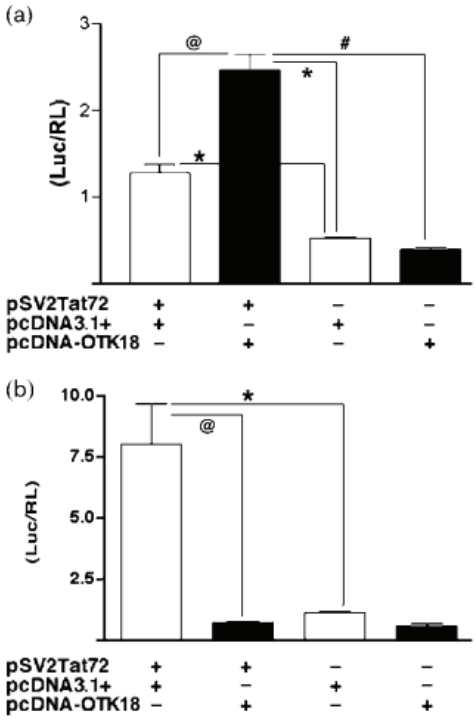

Figure 2. Luciferase assay of LTR elements and LTRΔEBS. (a) HEK293 cells were transfected with pSV2Tat72, pLTRΔEBS-Luc (firefly luciferase), pTK-RL (*Renilla* luciferase) and either pcDNA3.1 or pcDNA-OTK18. Transcriptional activity was expressed as a ratio of the reporter gene (pLTRΔEBS-Luc) to the reference gene (pTK-RL). (b) HXB2-derived original LTR-Luc was tested in the same experimental design. *, # and @ denote $P<0.05$ vs Tat(–) pcDNA3.1, Tat(–) pcDNA-OTK18 or Tat(+) pcDNA3.1, respectively.

The common Ets sequence was TGCATCCGGAG (89 % in A, 73 % in B, 3 % in C and 38 % in D), followed by TACATCCGGGAG (5 % in A, 4 % in B, 77 % in C and 13 % in D). The most striking difference is the specific dominance of TACATCCGGGAG in subtype C, which will be worthwhile to pursue for future study (the mutation TAC in type C is at position –149, whereas the beginning of the core Ets sequence is at –147). Further investigation is required to understand whether LTRs derived from different subtypes impact their suppression by OTK18 and if such an impact is attributed to the difference in the Ets sequence.

The upregulation of LTRΔEBS by OTK18 in the presence, but not in the absence, of Tat is unexpected, but it indicates that OTK18 may have a dual role in LTR regulation. We have found previously that there are two forms of OTK18 (Carlson *et al.*, 2004a), which we define as 75 kDa OTK18α and 65 kDa OTK18β. OTK18β lacks the KRAB-A box and may not act as a transcriptional suppressor. Thus, we believe that the dual gene regulation conferred by OTK18α and OTK18β may arise due to the presence or absence of different homology domains in these different OTK18 isoforms. Further study is necessary in order to characterize the expression of these OTK18 isoforms in MDMs and their respective roles in HIV-1 replication and LTR regulation.

Acknowledgements

We would like to thank Drs. A. Ghorpade and J. West for critical reading of the manuscript, the NIH AIDS Research and Reference Reagent Program for pSV2Tat72 (Alan Flankel) and LTR linker-scanning mutants (Steve Zeichner), S. Wakeley and G. Weber for technical assistance and R. Taylor for editorial support. This work is supported by NIH grants R01 AI5089401, R01 MH072539 (T. I.), K08 MH01552 (J. L.) and NCRR P20RR15635 (T. I. and C. J.).

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